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Genetic effects of persistent population bottlenecks on long-lived organisms with overlapping generations

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Genetic effects of persistent population bottlenecks on long-lived organisms with overlapping generations

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ABSTRACT

A population bottleneck is an event in which one population experiences a substantial reduction in number of individuals. The genetic consequences of bottlenecks include increased inbreeding, accelerated rate of random genetic drift, and decreased genetic diversity and adaptive evolution potential. These effects increase extinction probability and raise great concerns in conservation. Most previous theoretical work was developed under a simplifying assumption of discrete generations, thus creating complexities when applying the models to long-lived organisms. This study developed an overlapping-generation model to study the genetic consequence of bottlenecks in long-lived organisms. This model is implemented in a computer simulation program, BottleSim, to serve as a tool to evaluate the genetic consequences of bottlenecks. The first part of this study employs computer simulations to investigate the effects of generation model, longevity, reproductive system, and population size on the rate of decline in genetic diversity. The results suggest that each of these factors has a substantial effect on the rate of decline in genetic diversity during bottlenecks, and the traditional discrete-generation model tends to underestimate the rate. The second part of this study uses microsatellite markers to compare two ornate box turtle (*Terrapene ornata*) populations, one of which experienced a recent bottleneck due to habitat loss while the other is relatively undisturbed. The heterozygosity excess test detected the genetic signature of a recent bottleneck in the small population, but the bottleneck had little effect on the level of genetic diversity in this case. Based on life history attributes of this species and genetic projections made by computer simulations, a census population size of 700 is required for this imperiled population to maintain 90% of its observed allelic richness in the next 200 years. In conclusion, bottlenecks can have very different genetic effects on

long-lived species with overlapping generations and short-lived species with discrete generations. The life history of the organism must therefore be taken into account in practical conservation planning. This study developed a tool to facilitate conservation work involving long-lived species with overlapping generations, identified an imperiled ornate box turtle population, and provided conservation recommendations for this population.

CHAPTER 1.

General Introduction

Background

A population bottleneck is an event in which one population experiences a substantial reduction in population size (Hartl & Clark 1997). The reduced population size renders the population more susceptible to stochastic processes, thus increasing the short-term extinction risk (Lande 1993; Foose *et al.* 1995). In addition, population bottlenecks reduce genetic diversity, an important indicator for adaptive evolution potential, resulting in lower long-term survival probability (Avise 1995; Newman & Pilson 1997; Bouzat *et al.* 1998; Saccheri *et al.* 1998; Reed & Frankham 2003; but also see Reed & Frankham 2001). These increased short-term and long-term extinction probabilities caused by population bottlenecks raise great concerns in conservation (Seal *et al.* 1994; Gautschi *et al.* 2002).

Several different approaches have been employed in previous studies to better understand the genetic effects of population bottlenecks, including: (i) theory development, analytical equation derivation, and modeling (Wright 1931; Crow & Kimura 1970; Nei *et al.* 1975; Denniston 1977; Nunney & Elam 1994), (ii) computer simulations (Nei *et al.* 1975; Allendorf 1986; Lacy 1987; Luikart *et al.* 1998; England & Osler 2001), (iii) empirical study with experimental populations (Buri 1956; Spencer *et al.* 2000), (iv) empirical study with natural populations (Houlden *et al.* 1996; Friar *et al.* 2000; Akst *et al.* 2002; Malone *et al.* 2003), and (v) combinations of more than one approach (Hoelzel *et al.* 1993; Cornuet & Luikart 1996; Halley & Hoelzel 1996; Hoelzel 1999; Garza & Williamson 2001).

Challenges in the Field

In practical conservation planning, it is vital to integrate all the knowledge available to make biologically sound conservation plans that not only restore the imperiled populations demographically but also genetically (Quattro & Vrijenhoek 1989; Friar *et al.* 2000). In order to achieve this goal, integration of theoretical and empirical approaches is required. However, most theoretical work in the past has been based on many preset assumptions and/or "model" organisms to create simplified models (Lande 1995; Hartl & Clark 1997). This approach often generates difficulties for subsequently integrating theoretical and empirical work. One representative example is that virtually all mathematical and computer models in population genetics are based on the assumption of discrete generations (Crow & Kimura 1970; Watterson 1984; Allendorf 1986; Hartl & Clark 1997; Balloux 2001; England & Osler 2001; Garza & Williamson 2001; but also see Hill 1972; Lande 1995; Birnbaum *et al.* 2002). Though this assumption greatly simplifies the models and provides a good fit for short-lived insects and annual plants that have a short growing season, it creates complexities when applying the models to perennial plants and long-lived animals, which are important in many real world conservation cases (Lande 1995).

Goals and Values of the Study

To provide a solution to the problem stated above, the goals of this study are to: (i) develop an overlapping-generation model that provides a better fit for long-lived organisms with overlapping generations, (ii) implement the model in one easy-to-use computer program, and (iii) empirically test the model by using molecular markers to investigate populations of long-lived organisms with known demographic histories.

By achieving these goals, this study will: (i) provide a better understanding of the population genetic effects of bottlenecks on long-lived species with overlapping generations, (ii) create one freely distributed computer program that is useful for population and conservation geneticists, and (iii) provide genetic information on, and facilitate the conservation of, the populations involved in the empirical study.

Thesis Organization

Following this introduction, the first data chapter (Chapter 2) employs a computer simulation program (BottleSim, Appendix I) to investigate the genetic effects of persistent population bottlenecks on long-lived organisms with overlapping generations. Specifically, this chapter discusses the effects of reproductive lifespan length and reproductive system on the rate of decline in genetic diversity.

The second data chapter (Chapter 3) uses microsatellite markers to compare the genetic diversity in two natural populations of ornate box turtles (*Terrapene ornata*). The sequences of microsatellite primers used are reported in Appendix II. The two populations consisted of one small isolated population that experienced a recent bottleneck and one relatively undisturbed large population. Furthermore, the genotypic data from the small population are used as the input to a computer simulation program (BottleSim, Appendix I) to make future genetic forecasts for this imperiled population.

The conclusion chapter (Chapter 4) provides further discussion relevant to the two data chapters, examines major findings and values of this study, and suggests future research directions.

The Appendix I is a program note of the computer simulation program (BottleSim) developed in this study. A detailed description of the overlapping-generation model that is implemented in the algorithm is also provided in this appendix.

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CHAPTER 2.

Genetic Effects of Persistent Population Bottlenecks on Long-Lived Organisms with Overlapping Generations

Abstract

Expansion of human populations in the past few hundred years has caused population bottlenecks in many species. These persistent population bottlenecks reduce genetic diversity, and are a great concern in conservation genetics. We used computer simulations to investigate the effects of longevity and reproductive system on the rate of decline in genetic diversity. Notably, an overlapping-generation model is employed to provide a more realistic projection for long-lived organisms. The major findings are: (i) the overlapping-generation model is more suitable for making genetic projections in long-lived species, because the traditional discrete-generation model underestimates the rate of decline in genetic diversity, (ii) the severity of bottlenecks is the most important factor affecting the rate of decline in genetic diversity, and (iii) the length of expected longevity and the type of reproductive system considerably affect the rate of decline in genetic diversity. The results provide information concerning the genetic effects of persistent population bottlenecks on long-lived species in the time-scale of centuries, and the simulation algorithm should be a useful tool for conservation planning.

Keywords

Population bottleneck, genetic diversity, conservation genetics, computer simulation

Introduction

The rapid growth of human populations and the accompanying activities in the past few hundred years has caused substantial impacts on various ecosystems, threatening the long-term survival of many species. Extensive habitat fragmentation and overexploitation often cause persistent population bottlenecks in natural populations. Since the long-term survival of a population is largely dependent on its genetic diversity in addition to its demographic composition (Lande 1988; Avise 1995; Newman & Pilson 1997; Saccheri *et al.* 1998), the loss of genetic diversity due to persistent population bottlenecks is a great concern in conservation genetics (Frankel & Soule 1981).

To better understand the genetic effects of persistent population bottlenecks, mathematical modeling and computer simulations are often used (Crow & Kimura 1970; Allendorf 1986; Cornuet & Luikart 1996; Balloux 2001; England & Osler 2001; Garza & Williamson 2001). However, virtually all previous studies are based on a discrete-generation model, and employ the number of generations as the temporal unit (but also see Lande 1995; Birnbaum *et al.* 2002). This approach creates difficulties when applying theoretical and simulation studies to practical conservation planning. First, many organisms have overlapping generations, and the projections based on a discrete-generation model might not be realistic. Second, the number of generations might be difficult to estimate for organisms with overlapping generations, and thus raises complications when setting simulation parameters. Third, a discrete-generation model only provides estimates at the initiation of each new generation and leaves large gaps between generations when the organism in question has a long lifespan.

To alleviate these problems, we developed a computer simulation algorithm (BottleSim, Appendix I) that accommodates both discrete- and overlapping-generation models, and employs the number of years as the temporal unit. This algorithm is used as a tool to project the loss of genetic diversity under different scenarios. In the first part of this study, we investigate the effects of expected longevity on the rate of decline in genetic diversity. The second part of this study focuses on the effects of reproductive system on the rate of decline in genetic diversity. Ultimately, our goal is to have a better understanding of the genetic effects of persistent population bottlenecks on long-lived species in the time scale of centuries, and to provide a useful tool for conservation planning.

Materials and Methods

A computer algorithm that simulates the process of persistent population bottlenecks was used in this study (BottleSim, Appendix I). The parameters that remained constant throughout all simulations were as follows: (i) the observed number of alleles before the population bottleneck was set to 10, as is typically found in empirical microsatellite data (e.g. Roy *et al.* 1994; Houlden *et al.* 1996; Paetkau *et al.* 1997; DeWoody & Avise 2000; Cunningham *et al.* 2002; Tallmon *et al.* 2002), (ii) age of reproductive maturation was set to one, (iii) the population size before the bottleneck was set to 1000 to represent a large undisturbed population (Lynch 1996), (iv) the sex ratio was set to 1:1 in all simulations with a dioecious reproductive system, (v) the duration of the persistent population bottleneck was set to 200 years, as this represents a key period of increased human activities, and (vi) the number of iterations was set to 1000, as the values of mean and standard deviation are reasonably stable under this setting. The observed number of alleles and observed

heterozygosity are reported for each simulation, as these are the most representative and commonly used measurements of genetic diversity in empirical studies (e.g. Friar *et al.* 2000; Akst *et al.* 2002; Tallmon *et al.* 2002).

To investigate the differences between discrete- and overlapping-generation models, all simulations were performed under both models while other parameters remained unchanged. When using the overlapping-generation model, the degree of generation overlap was set to 100% (maximum overlapping). Two different population sizes during the bottleneck were chosen to represent a moderate bottleneck (population size = 100) and a severe bottleneck (population size = 20). Smaller population sizes were not simulated because a population with less than 20 individuals has a high extinction probability simply by stochastic processes (Lande 1993; Foose *et al.* 1995).

To evaluate the effect of longevity on the rate of decline in genetic diversity, four different settings were chosen (1, 5, 10, and 20 years). The reproductive system was set to dioecy with random mating, as this system is most representative of vertebrate populations and is commonly used in previous simulation studies (Allendorf 1986; Balloux 2001).

To investigate the effects of reproductive system on the rate of decline in genetic diversity, four reproductive systems were chosen (asexual reproduction, monoecy with selfing, dioecy with random mating, and dioecy with single reproducing pair). Two models of monoecy with random mating that are available in the BottleSim simulation algorithm were not included here as they produced very similar results to the model of dioecy with random mating. The model of dioecy with a single reproducing male was not included either, as it is intermediate between the random mating and single reproducing pair models.

Expected longevity of individuals was set to 10 years in all simulations that compared reproductive systems.

Results

Effect of expected longevity

The effect of expected longevity on loss of observed number of alleles and observed heterozygosity is summarized in figure 1 and figure 2, respectively. Observed number of alleles declined at a faster rate than observed heterozygosity (cf. Figs. 1 and 2), as found in previous theoretical and empirical studies (Nei *et al.* 1975; England & Osler 2001; Cunningham *et al.* 2002). The rate of decline in genetic diversity is affected by the population size and longevity as expected. In both cases, bigger is better: larger populations with longer lifespans lose genetic diversity slower than smaller populations with shorter lifespans. In addition, population size affects the number of observed alleles that a population could stably retain, regardless of the number of observed alleles at the initiation of the bottleneck (data not shown).

When the expected longevity equals the temporal unit used (i.e., the expected longevity equals one year), the discrete- and overlapping-generation models made identical projections (cf. bottommost lines in Figs. 1a and 1b, Figs. 1c and 1d, Figs. 2a and 2b, and Figs. 2c and 2d). But when the expected longevity is greater than one year, the projections made by the two models are quite different. The rate of decline in genetic diversity is much faster under the more biologically realistic overlapping-generation model for long-lived organisms (cf. Figs. 1a and 1c to 1b and 1d, respectively, and Figs. 2a and 2c to 2b and 2d, respectively).

Effect of reproductive system

The impact of reproductive system on loss of observed number of alleles and between observed number of alleles and observed heterozygosity depends on the reproductive system and has no general patterns (cf. Figs. 3 and 4). The generation model and population size affect the absolute rate of decline in genetic diversity, but do not affect the relative rates among different reproductive systems.

The asexual and random mating models are very similar in terms of decline in observed number of alleles, regardless of generation model or population size (Fig. 3). The single reproducing pair model represents an extreme case of reproductive skew, and has the fastest rate of decline in observed number of alleles, especially under the discrete-generation model (Figs. 3a and 3c). The monoecy with complete selfing model falls between the random mating and single reproducing pair model in most cases (Fig. 3a, 3b, and 3c), but loses observed number of alleles slightly faster than the single reproducing pair model when simulation parameters were set to a severe population bottleneck and overlapping generations (Fig. 3d).

Observed heterozygosity remained constant under asexual reproduction, while rapidly reaching zero under a selfing model, as expected (Fig. 4). The loss of observed heterozygosity under a random mating model is faster under the overlapping-generation setting than the discrete-generation setting (cf. Figs 4a to 4b and 4c to 4d). However, when reproductive skew exists (single reproducing pair model), the rate of decline in observed heterozygosity is faster under the discrete-generation setting, in contrast to the random mating and selfing models. Notably, population size affects the rate of decline in observed heterozygosity for the single reproducing pair model under the overlapping-generation

setting (cf. Figs. 4b and 4d), but has no effect under the discrete-generation setting (cf. Figs. 4a and 4c).

Discussion

As expected, the severity of population bottlenecks has a tremendous effect on the rate of decline in genetic diversity. The more severe the population bottlenecks, the faster the decline in genetic diversity. However, our study demonstrates that other factors such as the length of expected longevity, the type of reproductive system, and degree of generation overlap also have substantial influence on the rate of decline in genetic diversity. For practical conservation planning, none of these factors should be overlooked.

Expected longevity and generation models

Consistent with previous studies (Allendorf 1986; England & Osler 2001; Birnbaum *et al.* 2002), our results demonstrated that the most rapid decline in genetic diversity occurs in the first 10 to 40 generations, depending on the severity of the population bottleneck. When considering the time scale of 100 to 200 years, as often encountered in real world conservation situations, long-lived species often have relatively few population turnovers, and therefore lose significantly less genetic diversity than short-lived organisms as a result.

However, the simulations clearly demonstrated that the rate of decline in genetic diversity is much faster under the overlapping-generation model than under the discrete-generation model. Though counterintuitive at first sight, a reasonable explanation exists. Under the discrete-generation model, all individuals are expected to be involved in the mating process that reproduces the next generation, and a complete turnover of the

population occurs. This mode of reproduction assumes that each individual in the population has an equal genetic contribution to the next generation and, more importantly, each allele has the same transmission probability to the next generation. Alternatively, only a portion of the population is replaced every year under the overlapping-generation model. Individuals thereby have a lower probability to be chosen as the parents of the new individuals that replace them. The expected annual transmission probability for the alleles carried by the individuals being replaced is the reciprocal of reproductive lifespan. Though the cumulative genetic contribution throughout the reproductive lifespan is equivalent under both generation models, the overlapping-generation model has a higher probability of losing rare alleles when the individuals being replaced are the only carriers. As a consequence, observed number of alleles declines at a faster rate under the overlapping-generation model than under the discrete-generation model when the expected longevity remains constant. The same explanation also applies to the rate of decline in observed heterozygosity.

When comparing different reproductive lifespan settings under the overlapping-generation model, the result is more intuitive. The longer the expected longevity, the slower the rate of decline in genetic diversity. Though the annual transmission probability for the alleles carried by the individuals being replaced decreases as the reproductive lifespan increases, the extended reproductive lifespan reduces the rate of random genetic drift when using the number of years as the temporal unit. The reduced drift rate results in a lower probability that the individuals being replaced are the only carriers of rare alleles, and slows down the decline in genetic diversity.

Reproductive systems and generation models

Population bottlenecks have very different genetic effects on organisms with different reproductive systems. For example, organisms that are asexually reproducing or completely self-fertilizing would not experience any change in observed heterozygosity, regardless of the severity of the population bottlenecks, although observed number of alleles would be affected.

One notable finding of our simulations is that three different types of random mating models (monoecy and random mating with selfing, monoecy and random mating without selfing, and dioecy and random mating) all made very similar projections (data not shown). This result implies that the traditional random mating model should fit plant and animal systems equally well as long as the random mating assumption is fulfilled.

As we found previously, the loss of genetic diversity is more rapid under the overlapping-generation model than under the discrete-generation model for most reproductive systems. The only exception is when reproductive skew is present. In the extreme scenario we simulated (i.e., single reproducing pair each year), a population with discrete generations would soon reach fixation regardless of the observed number of alleles at the beginning. Since the founder pair reproduces all individuals of the next generation, the number of alleles at any locus would decline to four or fewer after only one generation. In contrast, only a portion of the population (i.e., the reciprocal of reproductive lifespan) is replaced every year under the overlapping-generation model, so the impact of reproductive skew on allelic richness is much less severe. This observation could reasonably explain how animals with a strong social reproductive hierarchy nonetheless maintain their genetic

diversity, since the dominant reproducing individuals account for a portion, but not all, of the surviving individuals in the next generation (Hoelzel *et al.* 1993; Hoelzel 1999).

Conservation implications

Though previous studies indicated that a population could experience an increase in quantitative genetic variability after a short-term population bottleneck (Bryant *et al.* 1986; Goodnight 1987; Lewin 1987), the persistent population bottlenecks caused by human activities would certainly diminish overall genetic variation in natural populations and reduce their evolutionary potential (e.g., Gautschi *et al.* 2002). For practical conservation planning, a clear understanding of the genetic effects of persistent population bottlenecks is vital. Our results unmistakably demonstrate that the discrete-generation assumption of previous theoretical studies could greatly underestimate the rate of decline in genetic diversity. Thus, the over-optimistic projections made under a discrete-generation model could hinder our ability to identify populations threatened by genetic impoverishment.

Valid reasons exist to keep theoretical modeling simple (Hartl & Clark 1997). However, certain oversimplifications make models significantly less realistic, and therefore difficult to apply to real world situations with great confidence. Many organisms have complex life histories that depart from the assumptions underlying simple theoretical models developed for model organisms (Lande 1995). Compared to an analytical approach, computer simulations can accommodate increased complexity of the model and incorporate various empirical data more readily. Considering the growing abundance of field data, the power of molecular tools, and the tremendous computational capacity available nowadays, realistic computer modeling based on complex empirical data is a reasonable expectation for

practical conservation planning. For example, a computer simulation algorithm that incorporates demographic data, multiple paternity rates, reproductive success, age-specific mortality and fecundity, allele frequency distributions, and other relevant empirical data will be a powerful tool for conservation geneticists. Certainly this integrative approach requires much effort in the field and laboratory, however, the projections would be much more realistic.

Both simple and complex modeling approaches have advantages and disadvantages, and the most sensible short-term solution may be a combination of the two approaches. Simple modeling would be appropriate for preliminary studies to identify populations that require active management. Caution should be taken due to the relative simplicity of the models; a conservative threshold is advised to reduce the possibility of missing true candidates for management. After the populations that require active management have been identified, the more complex modeling approach would then be appropriate for providing accurate genetic projections for practical conservation planning.

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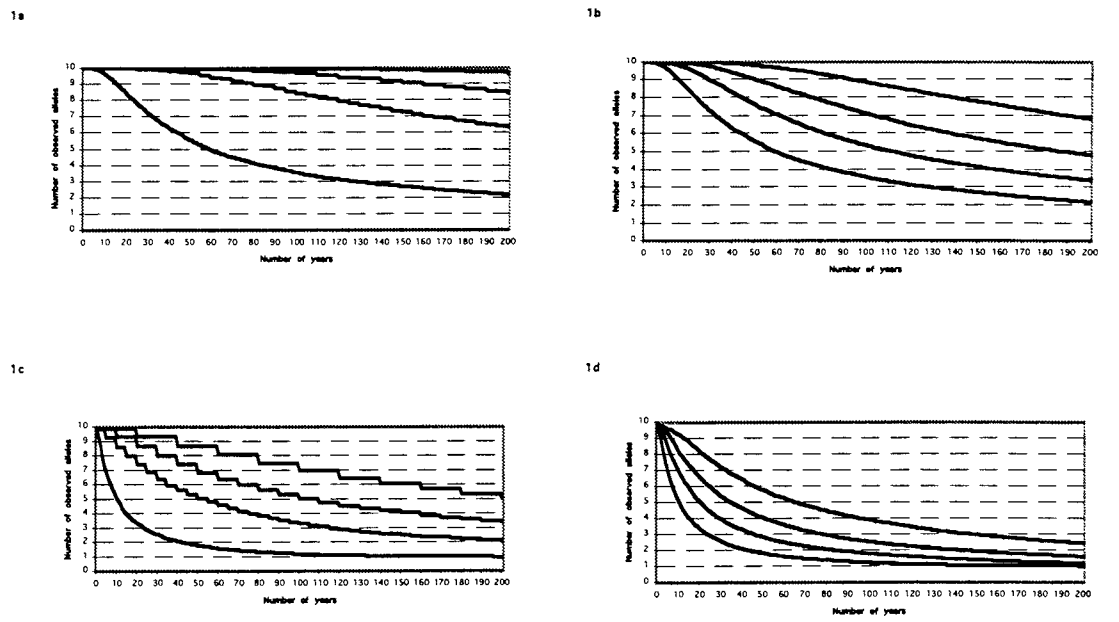

Fig. 1 Effects of expected longevity, bottleneck severity, and generation model on observed number of alleles. From bottom to top, curves show expected longevity of 1, 5, 10, and 20 years. a. Moderate population bottleneck (N = 100) under a discrete-generation model. b. Moderate population bottleneck (N = 100) under an overlapping-generation model. c. Severe population bottleneck (N = 20) under a discrete-generation model. d. Severe population bottleneck (N = 20) under an overlapping-generation model.

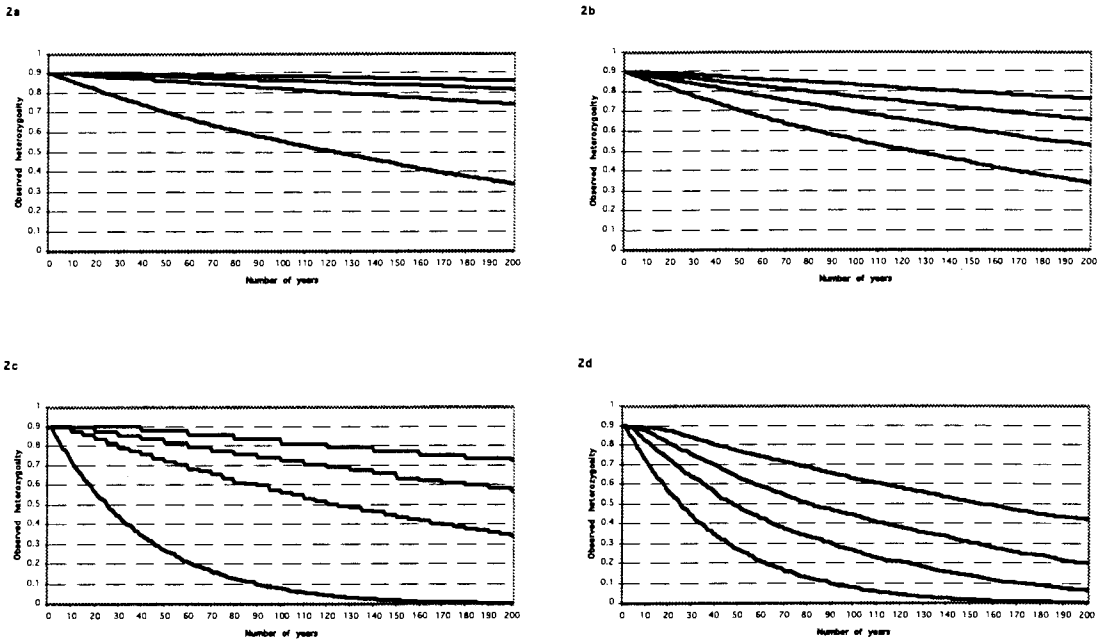

Fig. 2 Effects of expected longevity, bottleneck severity, and generation model on observed heterozygosity. From bottom to top, curves show expected longevity of 1, 5, 10, and 20 years. a. Moderate population bottleneck (N = 100) under a discrete-generation model. b. Moderate population bottleneck (N = 100) under an overlapping-generation model. c. Severe population bottleneck (N = 20) under a discrete-generation model. d. Severe population bottleneck (N = 20) under an overlapping-generation model.

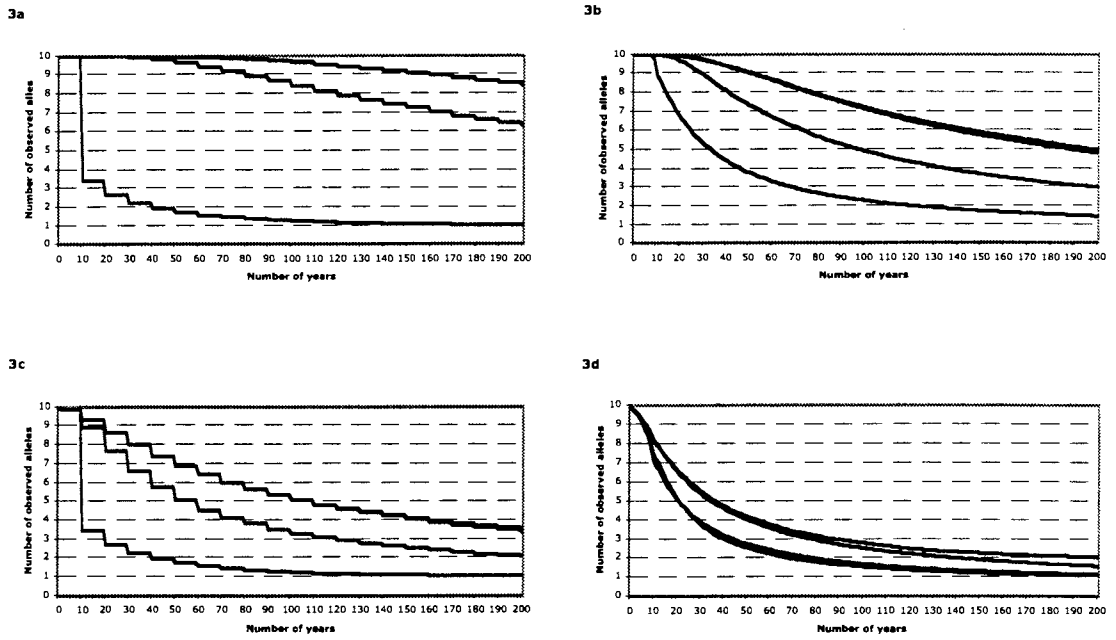

Fig. 3 Effects of reproductive system, bottleneck severity, and generation model on observed number of alleles. a. Moderate population bottleneck (N = 100) under a discrete-generation model. From bottom to top, curves show reproductive systems of dioecy with single reproducing pair, monoecy with selfing, and dioecy with random mating and asexual reproduction (overlapped). b. Moderate population bottleneck (N = 100) under an overlapping-generation model. From bottom to top, curves show reproductive systems of dioecy with single reproducing pair, monoecy with selfing, dioecy with random mating, and asexual reproduction. c. Severe population bottleneck (N = 20) under a discrete-generation model. From bottom to top, curves show reproductive systems of dioecy with single reproducing pair, monoecy with selfing, and dioecy with random mating and asexual reproduction (overlapped). d. Severe population bottleneck (N = 20) under an overlapping-generation model. From bottom to top, curves show reproductive systems of monoecy with

selfing, dioecy with single reproducing pair, dioecy with random mating, and asexual reproduction.

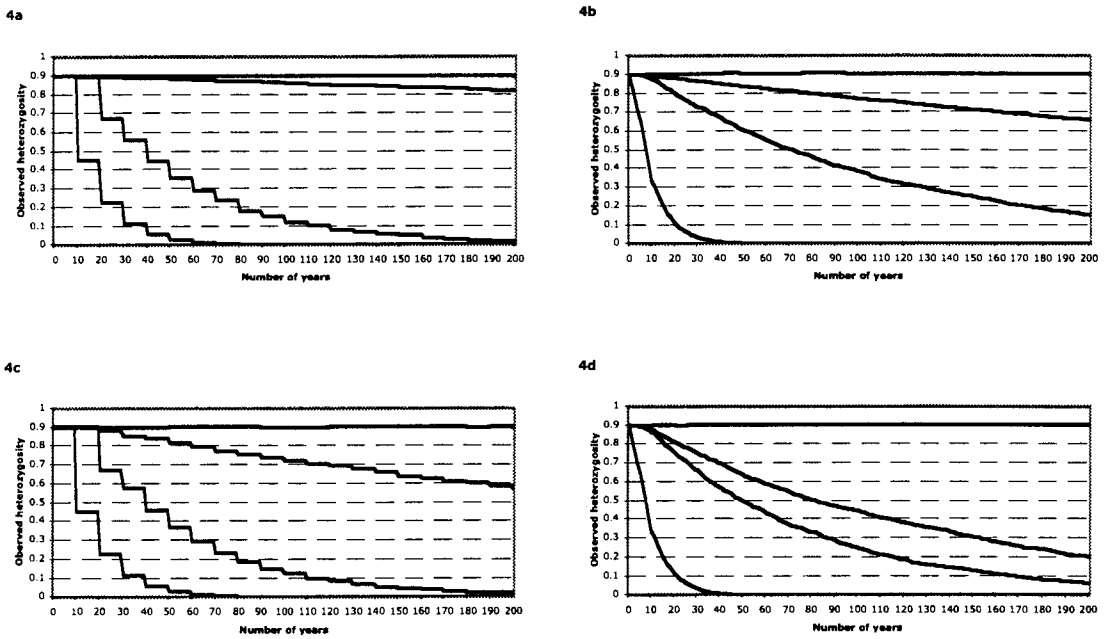

Fig. 4 Effects of reproductive system, bottleneck severity, and generation model on observed heterozygosity. From bottom to top, curves show reproductive systems of monoecy with selfing, dioecy with single reproducing pair, dioecy with random mating, and asexual reproduction. a. Moderate population bottleneck (N = 100) under a discrete-generation model. b. Moderate population bottleneck (N = 100) under an overlapping-generation model. c. Severe population bottleneck (N = 20) under a discrete-generation model. d. Severe population bottleneck (N = 20) under an overlapping-generation model.

CHAPTER 3.

Genetic Effects of a Persistent Bottleneck on a Natural Population of Ornate Box Turtles (*Terrapene ornata*)

Abstract

Human activities in the past few hundred years have caused enormous impacts on many ecosystems, greatly accelerating the rate of population decline and extinction. In addition to habitat alteration and destruction, the loss of genetic diversity due to reduced population size has become a major conservation issue for many imperiled species. However, the genetic effects of persistent population bottlenecks can be very different for long-lived and short-lived species when considering the time scale of centuries. To investigate the genetic effects of persistent population bottlenecks on long-lived species, we used microsatellite markers to assess the level of genetic diversity of a small ornate box turtle population that has experienced a population bottleneck in the past century, and compare this genetic diversity to that of a large relatively undisturbed population. The genetic signature of a recent bottleneck is confirmed by examining the deviation from mutation-drift equilibrium in the small population, but the bottleneck had little effect on the level of genetic diversity in this population. Computer simulations suggest that a census population size of 700 would be required for the small population to maintain 90% of its observed allelic richness in the next 200 years. The life history of long-lived species could mask the accelerated rate of genetic drift, making population recovery a relatively slow process. Statistical analysis of genetic data and empirical-based computer simulations can be important tools to facilitate conservation planning.

Keywords

Population bottleneck, conservation genetics, microsatellite, genetic diversity, *Terrapene ornata*

Introduction

Human populations have experienced rapid growth in the past few hundred years and greatly increased demand for natural resources. Many species have thus faced rapid extinction, while others have suffered from the loss of genetic diversity due to habitat fragmentation and overexploitation. Since genetic diversity is a primary component of adaptive evolution, the loss of genetic diversity seriously decreases the long-term survival probability of a population (Avise 1995; Newman & Pilson 1997; Bouzat *et al.* 1998a; Saccheri *et al.* 1998). For many imperiled species, active management plans are often required to prevent extinction. In order to make biologically sound conservation plans, understanding the genetic diversity remaining in natural populations is essential (Quattro & Vrijenhoek 1989; Friar *et al.* 2000), and is often investigated empirically by employing molecular markers (Haig 1998).

The ornate box turtle (*Terrapene ornata*) is a small terrestrial turtle that resides in sand prairie across the central and southern part of the United States and northern Mexico (Ernst *et al.* 1994; Dodd 2001). The arrival of European settlers and the accompanying agricultural and industrial activities in the Midwest U.S. destroyed a large fraction of the species' natural habitat, and restricted gene flow among remnant populations. In addition to habitat loss, the species is further threatened by collection due to its popularity on the pet market (Ernst *et al.* 1994). Habitat fragmentation, restricted gene flow, and reduced

population size all raise the concern that populations will become genetically impoverished, and thus unable to adapt to future environmental changes and to develop resistance to new diseases. The species is listed as endangered (IN, WI), threatened (IA), or other status of conservation concern (AK, CO, IL, KS, LA, MO, NE, OK), and is becoming rare even within its major distribution range (Levell 1997; Dodd 2001).

We used microsatellite markers to compare the genetic diversity of a small isolated population of *T. ornata* from fragmented habitat (IL) to a large relatively undisturbed population from major habitat (NE). The goal is to have a better understanding of human impacts on genetic diversity in remnant populations of this and similarly distributed species. In addition, we developed a computer simulation algorithm as a tool to forecast future genetic diversity under different scenarios. The simulation algorithm employs an overlapping-generation model that fits the study organism better than the traditional discrete-generation model. Ultimately, we want to present fundamental information for the conservation of the species, and also provide a useful computational tool for conservation studies involving long-lived species with overlapping generations.

Materials and Methods

Study populations and sample collection

Two ornate box turtle populations were sampled for the study. The small, isolated IL population has experienced a relatively recent population bottleneck due to habitat loss and human activities. Its current habitat is a sand prairie remnant of approximately 150 hectares, surrounded by the Mississippi River and heavily developed area (Carroll and Whiteside Counties, Illinois, USA). The development history of the surrounding area traces back for at

least 160 years (Davis 1993; Kett 1993), and our hypothesis is that the population size has been kept small since the beginning of this development. In addition, the population is further impacted by the increased automobile traffic and human collection in the past few decades (F. Janzen, personal observations). Blood samples were collected from 74 individuals (41 female, 27 male, 6 juvenile). We believe these samples include nearly all individuals in the current population based on our field observation and mark-recapture work since 1990.

The NE population is a large and relatively undisturbed population from major habitat (Valentine National Wildlife Refuge, Cherry County, Nebraska, USA), and is included in this study as the reference population. Blood or tail clip samples were collected by J. Kolbe from 73 individuals (sex was not identified for all individuals). Tissue samples were preserved in Queen's lysis buffer (Seutin *et al.* 1991) and stored at –80°C in the lab until DNA extraction.

Microsatellite genotyping procedure

Genomic DNA was extracted by using the High Pure PCR Template Preparation Kit (Roche), following the protocol provided by the manufacturer. We tested 16 microsatellite loci (appendix II) developed by Dr. Tim King (USGS) for the bog turtle (*Clemmys muhlenbergii*).

Reaction volume for the polymerase chain reaction (PCR) was 12.5 μL. The PCR mixture consisted of: 2 μL template DNA solution (with a concentration of approximately 100 ng/μL), 1.25 μL *Taq* DNA polymerase10X reaction buffer without $MgCl_2$ (Promega), 1.25 units of *Taq* DNA Polymerase (Promega), 4 mM $MgCl_2$, 100 nM of each dNTP (Takara), and 400 nM of each primer. Forward primers were labeled with fluorescent dye (6-FAM or TET) at the 5'-end. The PCR conditions were: one denaturing step at 94°C for 2

min, 34 cycles consisting of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, and a final elongation step of 72°C for 7 min. Samples were held at 4°C after the PCR amplification.

PCR products were diluted 1:2 with sterile water, and 1.5 μL of the diluted sample from each reaction was sent to the DNA Sequencing and Synthesis Facility at Iowa State University (Ames, IA, USA). Samples were run on an ABI 377 automated DNA sequencer with a ROX internal size standard (Applied Biosystem). Data files from the sequencer were analyzed with GeneScan v.3.0 and Genotyper v.2.0 software (Applied Biosystem). For quality control, three individuals with blood samples collected from different years were chosen to perform independent DNA extraction and genotyping.

Statistical analyses

Genotypic data were analyzed using POPGENE (Yeh & Boyle 1997) to calculate observed number of alleles, effective number of alleles, observed heterozygosity, and expected heterozygosity. The genetic divergence between populations was measured by F_{st} (Weir & Cockerham 1984; Weir 1996) and Analysis of Molecular Variance (AMOVA, Excoffier *et al.* 1992; Weir 1996), as implemented in ARLEQUIN (Schneider *et al.* 2000)

To test if the population bottleneck could be confirmed by molecular marker data, we used the program BOTTLENECK (Cornuet & Luikart 1996; Piry *et al.* 1999) to examine the deviation of genetic results from mutation-drift equilibrium. The two-phase model (TPM; Di Rienzo et al. 1994) was chosen because it is more appropriate than the strict one-step stepwise mutation model (SMM; Ohta & Kimura 1973) for microsatellite data (Piry *et al.* 1999). The parameters for TPM were set to 95% single-step mutations and 5% multiple-step

mutations, and the variance among multiple steps was set to 12 as suggested by Piry *et al.* (1999). To assess the robustness of this test, we performed additional tests with different parameter settings. The single-step mutation rate in additional tests ranges from 90% to 99%, the variance among multiple steps ranges from 2 to 24. Based on the number of loci in our data set, the Wilcoxon test was chosen for the statistical analysis of heterozygote excess.

Computer simulations

To forecast the future genetic diversity level for the IL population and to make recommendations regarding sustainable population size, we developed a computer program that simulates the process of random genetic drift (BottleSim; Appendix I). The simulation algorithm generates hypothetical populations of fixed size based on the allele frequencies calculated from input genotypic data to project genetic diversity decline due to random genetic drift.

Three sets of simulations were performed to forecast the decline in genetic diversity under different scenarios: (i) actual allele frequencies using the overlapping-generation model, (ii) equal allele frequencies using the overlapping-generation model, and (iii) actual allele frequencies using the discrete-generation model. The first set used the actual allele frequency data from the IL population and the overlapping-generation model to provide the most realistic projections. To estimate the effects of allelic distribution skew in the IL population, a second set of simulations was performed with an input file that had identical observed allelic richness with the IL population but equal allelic distribution at each locus (i.e. each allele at the same locus had equal frequency). The model was again set to overlapping generations in this second set of simulations. To explore the differences between

the overlapping- and discrete-generation models, the third set of simulations was performed with empirical allele frequencies from the IL population under the discrete-generation model. All simulation parameters except population size and generation model remained constant and were set as follows: degree of generation overlap = 100 when using the overlapping-generation model (i.e. all individuals start with a random age value that is within the longevity limit), dioecy with random mating reproductive system, expected longevity = 30 years, age of reproductive maturation = 10 years, male:female ratio was set to 1:1.5 based on our sex ratio data from the IL population and previous studies of other ornate box turtle populations (Legler 1960; Doroff & Keith 1990), number of years simulated = 200 years, and number of iterations = 1000.

Previous studies indicate that the ornate box turtle has a relatively long lifespan in the wild and in captivity. Legler (1960) expected the species could live up to 50 years in the wild and Blair (1976) estimated the species has a lifespan of approximately 30 years based on 23 years of field studies. Metcalf and Metcalf (1985) reported an average life span of approximately 22 years from their 26 years of field studies. Ernst (Ernst *et al.* 1994) reported a record of 42 years for a female box turtle that lived 22 years in captivity. The species typically reaches reproductive maturation at the age of 7-11 years (Legler 1960; Blair 1976). Consequently, we set the expected longevity to 30 years and the reproductive maturation age to 10 years in all our simulations.

Results

Genetic diversity, genetic divergence, and BOTTLENECK test

All 16 microsatellite primer pairs developed for the bog turtle successfully amplified ornate box turtle DNA. Two loci were found to be duplicated in the ornate box turtle genome (CmuB21 and CmuD62) and three loci were found to be monomorphic in both study populations (CmuB67, CmuB91, and CmuD93). The five loci above were excluded from further analyses. The genotyping results were perfectly repeatable for all loci for the three individuals with independent blood samples collected from different years.

The IL and NE populations possessed relatively similar metrics of allelic richness and heterozygosity (Tables 1 and 2). The IL population had one more polymorphic locus than the NE population (CmuD79). The average observed number of alleles was about 7.5 in the IL population and 8 in the NE population; the average effective number of alleles was 4.7 in the IL population and 4.3 in the NE population. No significant difference was found between populations for either allelic richness measurement ($P > 0.05$ for both t-tests). Private alleles accounted for nearly 32% of the total alleles found (33/102, 14 from the IL population and 19 from the NE population). The mean observed heterozygosity was 0.5615 in the IL population and 0.5730 in the NE population; the mean expected heterozygosity was 0.6743 in the IL population and 0.6860 in the NE population. Differences between the two populations for both measurements were not significant ($P > 0.05$ for both t-tests).

Despite similar metrics of genetic diversity, the two populations exhibited significant genetic divergence. The F_{st} value was 0.09891 and was highly significant ($P < 0.00001$). This F_{st} value indicates moderate genetic differentiation according to Wright's qualitative guideline for the interpretation of F_{st} (Wright 1978) and is at the lower end of the range

(0.011-0.631) found in other reptile studies using microsatellite markers (Ciofi & Bruford 1999; Ciofi *et al.* 2002; Cunningham *et al.* 2002; Dever *et al.* 2002; Beheregaray *et al.* 2003; Malone *et al.* 2003). However, one should note that this study only included two populations. AMOVA indicated that within-population variation in these microsatellite loci accounted for 90.11% of the total variation found.

The heterozygosity excess test implemented in the BOTTLENECK program was suggestive in the IL population (P = 0.05), but not significant in the NE population (P = 0.54). In the additional tests that assess the robustness of this test, the P values ranges from 0.03 to 0.10 in the IL population and from 0.81 to 0.38 in the NE population. In other words, the test of mutation-drift equilibrium tentatively supports a relatively recent population bottleneck in the small IL population, but not in the large NE population.

Simulation

Tables 3 and 4 summarize simulation projections of observed number of alleles and observed heterozygosity retained after a 200-year period compared to the current level. Observed number of alleles declined at a faster rate than observed heterozygosity, as found in previous theoretical and empirical studies (Nei *et al.* 1975; England & Osler 2001; Cunningham *et al.* 2002). Based on the actual allelic distribution, the IL population would retain about 71% of the observed number of alleles and 91% of the observed heterozygosity if its population size remained constant at 75 for the next 200 years. In order to achieve the conservation recommendation of maintaining 90% of genetic diversity for a 200-year period (Soule *et al.* 1986), the population size would need to be increased to approximately 300.

In comparison, less than 150 individuals are required to maintain 90% of observed number of alleles if the population started with an equal allelic distribution. The skewed allelic distribution of the IL population resulted in a faster rate of decline in observed number of alleles. The probability of losing a rare allele due to random genetic drift increases as its frequency decreases.

Notably, the projections of genetic diversity are quite different under the overlapping-generation model and the discrete-generation model. The rate of decline in genetic diversity is much faster under the more realistic overlapping-generation model. To maintain 90% of observed allelic richness under the discrete-generation model for 200 years, a population size of 150 is sufficient. This number is considerably smaller than the 300 suggested by the overlapping-generation model.

Discussion

Our study suggests that remnant populations of long-lived species might appear to be genetically healthy, even after a persistent population bottleneck that lasts for 100 to 200 years. This could have rather complex implications for long-lived species. On the negative side, the accelerated rate of genetic drift in such populations could be masked and thus could be overlooked for necessary conservation efforts. On the positive side, the genetic diversity still present permits development of proper conservation plans that could restore populations not only demographically, but also genetically. This genetic situation is analogous to the demographic situation facing long-lived organisms where adults are targeted for removal. Delayed sexual maturity of these organisms provides a juvenile pool that can buffer the

chronic disturbance, but can also mask the inevitable population extinction (Congdon *et al.* 1993; Congdon *et al.* 1994; Heppell 1998).

The two study populations of ornate box turtles exhibited the same level of genetic diversity for all four measurements we employed. This result might indicate that the two populations have approximately the same effective population size historically. The heterozygosity excess test developed by Cornuet and Luikart (1996) examines if the population in question deviates from mutation-drift equilibrium, and is useful for detecting historical population bottlenecks (Luikart & Cornuet 1998; Luikart *et al.* 1998a; Luikart *et al.* 1998b; Spencer *et al.* 2000). Our microsatellite data tentatively support the hypothesis based on demographic data that the IL population experienced a recent bottleneck and that the NE population did not. The additional BOTTLENECK tests with different parameter settings verified that this result was not very sensitive to the parameter settings. This recent and ongoing bottleneck experienced by the IL population apparently did not affect the level of genetic diversity represented by the microsatellite loci we analyzed. This surprising result might be caused by the long lifespan of this species and/or the severity of the bottleneck.

Computer simulations indicate that a hypothetical population that has the same level of observed allelic richness as the IL population but equal allelic distribution could maintain 90% of its observed allelic richness for 153 years with a constant population size of 75. This finding might explain why the small IL population maintains the same level of genetic diversity as the large NE population. Considering that the actual population decline for the IL population could be a gradual process rather than a sudden crash, we might expect that the IL population has maintained most of its historical genetic variation before the bottleneck.

The relatively long lifespan of ornate box turtles might also explain the similarity in allelic composition between the two populations. Though the two populations have probably been separated for thousands of years, the generation number since last exchanging migrants could be comparatively low. Phylogeographic studies of mtDNA in other turtle species (e.g., Weisrock & Janzen 2000; Starkey *et al.* 2003) suggest that current populations in the Midwest originated from the southern U.S. and that dispersal occurred after the last glaciers retreated about 10,000 years ago. The allele set found in both populations might, then, simply be representative of the ancestral southern U.S. gene pool.

Our hypothesis to explain the small but significant F_{st} value and the among-population variation is that the two populations were not significantly divergent before the human impacts occurred. In other words, we hypothesize that the recent population bottleneck in the IL population accelerated the drift rate in the past century, and resulted in this significant F_{st} value and among-population variation. Because the bottleneck was probably not severe, both the F_{st} and among-population variation estimates are small compared to other studies (e.g., Ciofi & Bruford 1999; Ciofi *et al.* 2002; Dever *et al.* 2002; Beheregaray *et al.* 2003; Malone *et al.* 2003).

The rate of random genetic drift in *T. ornata* is relatively slow when measured by the temporal unit of year, but this fact certainly does not make the species immune to the process when the population size declines. Our simulation results demonstrated that the IL population would lose 30% of its observed allelic richness in the next 200 years if the population size remained at 75 (Table 3). To maintain 90% of the observed allelic richness over a 200-year period, as recommended by Soule *et al.* (1986), a population size of 300 is required. One cautionary finding of our simulation study is that only 150 individuals are required to

maintain the same level of genetic diversity under a traditional discrete-generation model (Table 3). Considering that the overlapping-generation model is more realistic for long-lived organisms such as turtles and large mammals, this important factor must be taken into account when making conservation plans for such long-lived organisms.

Another important consideration is that the simulation model is based on several ideal population assumptions (complete random mating, constant population size, no selection, no migration, no mutation), thus the "population size" in this simulation model should be viewed as effective population size. Direct use of the population size guideline obtained from the simulations in practical conservation planning may not be appropriate. Effective population size is difficult to estimate in natural populations (Pope 1996) and the ratio of effective population size to census population size (N_e/N) can vary widely depending on life history of the organism in question (Nunney 1995). Previous studies found that for long-lived species with overlapping generations, effective population size is generally close to half of the breeding adult number (Nunney 1991; Nunney 1993; Nunney & Elam 1994). We found that adults account for approximately 92% of the IL population and Legler (1960) reported a Kansas population consisted of 84% adults. Based on the simulation results and population structure information, a census population size of 700 is therefore desirable for the IL population to maintain 90% of its observed allelic richness in the next 200 years.

The simulation results agree well with the 50/500 conservation rules laid out by Franklin (1980). Using an effective population size of 50, the IL population can still maintain 90% of observed allelic richness for 36 years and 90% of observed heterozygosity for 167 years. This result indicates that maintaining an effective population size of 50 can be a short-term solution to minimize inbreeding depression for the species. When effective population

size is increased to 500 in the simulation, the IL population can maintain 94% of observed allelic richness and 99% of observed heterozygosity for 200 years. This result suggests that maintaining an effective population size of 500 can effectively minimize the depletion of genetic variance for a population of this species.

The female-biased adult sex ratio that we found in the IL population is consistent with previous studies of ornate box turtle populations. Legler (1960) reported a 1:1.7 male:female ratio from a population in Kansas, whereas Doroff and Keith (1990) reported a 1:1.56 male:female ratio from a population in Wisconsin. These findings lead us to believe that this biased adult sex ratio might be the norm in this species, and we used this number to set the sex ratio parameter in all our simulations. However, this biased sex ratio only reduces the effective population size to 96% of the census size and has little effect on drift rate. The simulation results under 1:1 and 1:1.5 male:female ratio were not significantly different (data not shown).

Previous studies indicated that the population density of ornate box turtle populations is 2.9-13.1 adults/ha (Legler 1960; Doroff & Keith 1990). This finding suggests that the current 150-ha habitat of the IL population might be adequate to support the desirable census population size of 700. Previous work identifies adult survival as vital in the management of declining populations of long-lived organisms (Congdon *et al.* 1993; Congdon *et al.* 1994; Heppell 1998; Belzer 2002). Mortality of the species is high at juvenile stage, but becomes low when the animals reach maturation: survivorship of adults is estimated at 81-96%/year (Blair 1976; Metcalf & Metcalf 1985; Doroff & Keith 1990). However, automobiles are the major threat to these animals in developed areas, killing more adults than all predators

combined (Ernst *et al.* 1994; Gibbs & Shriver 2002). Consequently, fences may be required as well to keep the turtles from surrounding roads.

Several additional factors make the population recovery of ornate box turtles difficult without active management, such as slow maturity, high mortality for juveniles, and the potential threat from pet trade collectors. The species has a low intrinsic population growth rate, thus utilizing individuals from large healthy populations in recovery plans of imperiled populations might be a reasonable option. The genetic divergence data indicated that the IL population and NE population have similar allelic compositions, and the among-population variation only accounted for a small portion of the total variation found. This genetic similarity suggests that the NE population could be a candidate for helping the recovery of the IL population, if no other populations that are more appropriate could be found. The population size of the IL population can be increased either through the contribution of eggs (preferable) or translocation of adult individuals (less desirable because of potential disease and homing issues) from other populations (Dodd & Siegel 1991; Moore 1993).

In addition to rendering populations more susceptible to environmental stochasticity (Lande 1993; Foose *et al.* 1995), bottlenecks also reduce allelic richness and heterozygosity (Houlden *et al.* 1996; Bouzat *et al.* 1998b; Akst et al. 2002; Cunningham *et al.* 2002). Organisms with different life histories and reproductive systems could experience different rates of decline in genetic diversity. However, this decline would inevitably lead to a decrease in fitness (Gautschi *et al.* 2002; Beheregaray *et al.* 2003) and an increase in extinction probability (Frankham 1998; Saccheri *et al.* 1998). Our study demonstrates that long-lived organisms have a relatively slow rate of decline in genetic diversity, but are not immune to the process. The long lifespan of these species can be a double-edged sword. On

the one hand, it considerably slows down the rate of drift; on the other, it also makes the recovery process relatively slow. Careful investigations are needed to identify populations that appear to be genetically healthy but have an accelerated drift rate that is masked by long lifespan. Computer simulations based on empirical data could be a useful tool to help conservation planning in this regard. Conservation will be much more difficult when populations actually become genetically impoverished, and is much more effective and easy to implement when the populations are still genetically healthy.

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	IL		NE		All	
Locus	OA	EA	OA	EA	OA	EA
CmuA19	10	5.7051	9	2.4309	13	4.6063
CmuB08	4	2.3372	7	4.2769	7	3.3910
CmuB12	2	1.2717	3	2.3669	3	1.8261
CmuD21	4	2.8353	6	3.2112	6	3.3651
CmuD55	10	6.3140	11	6.7370	13	7.4623
CmuD79	2	1.4601	1	1	2	1.2163
CmuD87	12	7.1291	14	7.0583	15	10.4107
CmuD88	10	7.5858	10	5.2463	13	8.5830
CmuD90	9	6.9298	9	5.4756	9	7.3058
CmuD95	11	7.7226	8	5.7579	11	8.5162
CmuD121	9	1.9902	10	3.4374	10	2.7520
Mean	7.5455	4.6619	8	4.2726	9.2727	5.4032
St. Dev	3.7514	2.6569	3.6606	1.9492	4.3149	3.1453

Table 1. Observed number of alleles (OA) and effective number of alleles (EA).

	IL		NE	
Locus	H_o	H_e	H_o	H_e
CmuA19	0.8382	0.8308	0.4861	0.5928
CmuB08	0.5811	0.5760	0.8493	0.7715
CmuB12	0.2162	0.2151	0.5616	0.5815
CmuD21	0.6438	0.6518	0.6986	0.6933
CmuD55	0.4658	0.8474	0.7808	0.8574
CmuD79	0.2838	0.3172	0.0000	0.0000
CmuD87	0.8767	0.8657	0.7671	0.8642
CmuD88	0.6575	0.8742	0.4571	0.8152
CmuD90	0.7123	0.8616	0.4348	0.8233
CmuD95	0.4286	0.8768	0.5915	0.8322
CmuD121	0.4730	0.5009	0.6761	0.7141
Mean	0.5615	0.6743	0.5730	0.6860
St. Dev	0.2112	0.2420	0.2351	0.2483

Table 2. Observed heterozygosity (H_o) and expected heterozygosity (H_e).

Population size	Actual allele frequency, overlapping-generation model		Equal allele frequency, overlapping-generation model		Actual allele frequency, discrete-generation model	
50	4.5076	(63.5464)	5.5337	(73.3430)	5.6409	(79.6002)
75	5.1750	(70.9160)	6.3770	(84.5145)	6.1724	(84.7524)
150	6.1341	(82.0365)	7.3178	(96.9831)	6.8467	(91.5571)
200	6.4562	(86.0042)	7.4743	(99.0566)	7.0432	(93.7477)
300	6.8128	(90.3740)	7.5369	(99.8867)	7.2541	(96.2243)
500	7.1543	(94.8237)	7.5454	(99.9988)	7.4210	(98.3625)

Table 3. Observed number of alleles retained over a 200-year period based on computer simulations. Values in parentheses are the percentages retained (year 200/year 0).

Population size	Actual allele frequency, overlapping-generation model		Equal allele frequency, overlapping-generation model		Actual allele frequency, discrete-generation model	
50	0.5877	(87.8149)	0.7007	(87.5449)	0.6360	(95.1047)
75	0.6141	(91.7135)	0.7304	(91.2574)	0.6473	(96.6901)
150	0.6401	(95.5556)	0.7649	(95.5271)	0.6580	(98.2256)
200	0.6468	(96.6540)	0.7737	(96.6315)	0.6604	(98.6935)
300	0.6534	(97.6048)	0.7825	(97.7851)	0.6641	(99.1357)
600	0.6614	(98.7779)	0.7897	(98.6782)	0.6661	(99.4423)

Table 4. Observed heterozygosity retained over a 200-year period based on computer simulations. Values in parentheses are the percentages retained (year 200/year 0).

CHAPTER 4.

General Conclusions

General Discussion

Reduction of population size and genetic diversity caused by bottlenecks increases the probability of extinction (Avise 1995; Newman & Pilson 1997; Bouzat *et al.* 1998a; Saccheri *et al.* 1998). In practical conservation, the bottlenecks experienced by many imperiled populations are caused by human activities (Hoelzel 1999; Bouzat *el al* 1998b), and active management may be required to prevent extinctions of these populations (Ballou *et al.* 1994). Evidence demonstrates that the level of population genetic diversity often positively correlates with fitness (Quattro & Vrijenhoek 1989; Bouzat *et al.* 1998a; Coltman *et al.* 1998; Frankham 1998; Saccheri *et al.* 1998; Reed & Frankham 2003; but also see Reed & Frankham 2001), suggesting the importance of restoring imperiled populations not only demographically but also genetically (Avise 1995; Taylor & Dizon 1996; Friar *et al.* 2000; Tallmon *et al.* 2002). Consequently, levels of genetic diversity existing in imperiled and undisturbed populations have to be investigated empirically as the first step following the identification of imperiled populations (Friar *et al.* 2000; Cunningham *et al.* 2002). Advances in molecular biology in the past few decades have provided numerous invaluable molecular markers for this purpose (O'Brien 1994; Haig 1998).

The levels of genetic diversity in natural populations are affected by many factors (Amos & Harwood 1998), and clearly the life history and reproductive system are very important ones (Bourke *et al.* 1997; Hoelzel 1999). Most previous theoretical work has been based on many assumptions that simplify the mathematical models, such as random mating

and discrete generations. Though these simplifications provide a good starting point for theoretical work and encompass the biological reality of some organisms, the eternal pursuit of intellectual challenges and the practical values that drive scientific research both elicit the need to generate more intricate models that accommodate organisms with complex life histories. The tremendous computational capacity accessible to researchers and the growing abundance of empirical data present an exciting opportunity to make joint advancements in the fields of population and conservation genetics.

Major Findings and Values of the Study

The major findings of this study are: (i) the overlapping-generation model is more suitable for making genetic projections in long-lived species, because the traditional discrete-generation model underestimates the rate of decline in genetic diversity, (ii) the severity of bottlenecks is the most important factor affecting the rate of decline in genetic diversity, (iii) the longevity and the type of reproductive system considerably affect the rate of decline in genetic diversity, short lifespan and deviation from random mating both increase the rate of decline in genetic diversity, (iv) populations of long-lived organisms could retain most of their genetic diversity even after a persistent bottleneck of relatively long duration, and (v) the long lifespan of long-lived organisms could mask an accelerated rate of genetic drift and slow down genetic recovery from a bottleneck, thus cautious investigation is required.

This study contributes to the fields of population and conservation genetics by: (i) improving knowledge of the genetic effects of persistent population bottlenecks on long-lived species with overlapping generations, (ii) identifying an imperiled ornate box turtle

population and providing restoration recommendations, and (iii) creating one freely available and easy-to-use computer program that can facilitate practical conservation planning.

Future Research Recommendations

The simulation model developed in this project predicted that the two focal ornate box turtle populations would not differ much in terms of allelic richness and heterozygosity. This prediction is confirmed by the empirical microsatellite data. However, this result might only provide half of the support required to validate the simulation model. To gain the other half of the support needed, further empirical studies using populations predicted to have different levels of genetic diversity are necessary. Due to the difficulties involved with natural populations, such as uncertainties of population histories and sample collection, experimental populations could be a reasonable alternative. More importantly, experimental populations would allow statistical designs that validate the model quantitatively, which would be extremely difficult to achieve by using natural populations. Several organisms commonly used in evolutionary experiments that have a short generation time but overlapping generations, such as fruit fly (*Drosophila*), flour beetle (*Tribolium*), sand cricket (*Gryllus*), mosquitofish (*Gambusia*), zebrafish (*Danio*), and *Arabidopsis*, could be good candidates for generating experimental populations

Once the simulation model is validated empirically, the model can be expanded to provide more accurate projections. Three illustrative examples are to incorporate mutation, migration, and selection, as these three fundamental evolutionary processes could have substantial influences on the level of genetic diversity (Wade & Goodnight 1998; Neff & Gross 2001; Ingvarsson 2002). Other potentially useful expansions of the model would be to

incorporate age-specific mortality and fecundity, seed bank, sperm storage, and multiple paternity.

Future empirical studies in this field will improve our understanding of the genetic effects of bottlenecks and provide the foundation for evolution theory and model development. The modeling and development of computer algorithms will provide conservation geneticists with powerful tools to facilitate conservation planning. All will offer the combination of intellectual challenges and practical values that many scientists are increasingly pursuing.

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APPENDIX I.

BottleSim: a Bottleneck Simulation Program for Long-Lived Species with Overlapping Generations

Abstract

Population bottlenecks reduce genetic diversity and thus cause great concern in conservation biology. Previous theoretical studies often assume discrete generations in projecting declines in genetic diversity caused by bottlenecks. This assumption creates complexities when applying the models to long-lived species with overlapping generations. BottleSim is a program for simulating bottlenecks to estimate the impact on genetic diversity; the novelties include an overlapping-generation model, a wide range of reproductive systems, and flexible population size settings. With these features, BottleSim will be a useful tool for estimating the genetic consequences of bottlenecks, evaluating conservation plans, and performing power analysis.

Keywords

Population bottleneck, long-lived species, conservation genetics, allelic diversity, heterozygosity, computer simulation

Text

Population bottlenecks reduce genetic diversity and increase the possibility of population extinction (Lande 1994; Mills & Smouse 1994; Frankham 1995; Lynch *et al.* 1995). Major characteristics of population bottlenecks in conservation studies include: (i)

caused by human activities, such as overexploitation and habitat fragmentation, (ii) the duration of the bottleneck can be estimated by referencing to human activity history, and (iii) will most likely persist unless restoration work is implemented (Ballou *et al.* 1994).

Genetic diversity is an important index for estimating a population's long-term survival possibility (Frankel & Soule 1981), and is required for making biologically sound conservation plans (Friar *et al.* 2000). Common measurements of genetic diversity include average number of alleles per locus (observed and effective), heterozygosity (observed and expected), and the proportion of polymorphic loci (Frankel & Soule 1981). Molecular markers are important modern tools for estimating the level of genetic diversity in imperiled populations. To estimate the loss of genetic diversity in imperiled populations, results are often compared to empirical values obtained from undisturbed populations and theoretical projections.

While the loss of heterozygosity is described in previous theoretical studies (Crow & Kimura 1970), the loss of allelic diversity is difficult to estimate for more than one generation (Denniston 1977; Watterson 1984). Due to the difficulty of estimating allelic diversity loss, computer simulations are often used for making projections (Nei *et al.* 1975; Allendorf 1986; England & Osler 2001).

The vast majority of previous studies assumed discrete generations, and employed the number of generations as the temporal unit for measuring decreases in genetic diversity (Wright 1969; Crow & Kimura 1970; Watterson 1984; Allendorf 1986; Garza & Williamson 2001; but also see Hill 1972; Lande 1995). Though this assumption greatly simplifies the models and is the biological reality of many short-lived species, it creates difficulties when applying the theoretical and simulation studies to practical conservation planning. First,

many organisms have more complex life histories, often with overlapping generations. In these situations, simulations based on the discrete-generation model might not be realistic. Second, the number of generations might be difficult to estimate for organisms with overlapping generations and causes problems when setting simulation parameters. Third, the point estimations made by a discrete-generation model leave large gaps between generations for long-lived species. Most of the simulation programs available to population geneticists are designed under a discrete-generation model (e.g., EASYPOP (Balloux 2001), GENELOSS (England & Osler 2001)); for the ones that accommodate overlapping generations (e.g., MANAGEDPOP (Birnbaum *et al.* 2002), METASIM (Strand 2002)), constraints on population size settings render those programs unsuitable for simulating population bottlenecks.

BottleSim is a program specifically designed for simulating the genetic consequences of bottlenecks and post-bottleneck population growth for long-lived species. In addition to an overlapping-generation model, BottleSim allows users to specify an arbitrary population size each year. This feature enables users to simulate a wide range of scenarios (e.g., gradual versus rapid population decline, repeated bottlenecks, exponential versus logistic population growth). The capability of using an arbitrary population size each year also eliminates the need to calculate effective population size when population size fluctuates. Though the harmonic mean of successive generations generally provides a good approximation of effective population size (Nei *et al.* 1975), significant deviations can occur if populations experience cyclic changes in size (Motro & Thomson 1982).

BottleSim also provides a wide range of reproductive system settings. The reproductive system can be set to asexual, monoecious (strict selfing, random mating with

selfing, and random mating without selfing), or dioecious (random mating, single reproducing male each year, and single reproducing pair each year). Sex ratio can be specified by users when a dioecious reproductive system is chosen. In cases where reproductive skew is simulated, a single reproducing male or pair is randomly chosen from the population each year and reproduces all the new individuals in that year. With these options and those involving overlapping generations and population size, BottleSim is expected to be a useful tool for population and conservation geneticists.

The current version (version 2.6) of BottleSim includes four simulation modules (single locus with constant population size, single locus with variable population sizes, multilocus with constant population size, and multilocus with variable population sizes). The diploid single locus modules accept arbitrary allele frequencies at a single locus and are intended for exploring the genetic consequences of population bottlenecks under different settings. The diploid multilocus modules accept multilocus genotypic data of codominant markers, and are useful for evaluating conservation plans of different population sizes based on the empirical genotypic data. The input files must be ASCII text files (e.g. MS-DOS text or text-only); the input file format is described in detail in the users' manual.

User-defined parameters for simulations include the degree of generation overlap (0-100%, 0 = discrete generations, 100 = completely overlapping generations), reproductive system, expected longevity of the organism, age of reproductive maturation, population sizes, the number of years to simulate, and the number of iterations. Observed number of alleles and allele frequencies in the founder population can be specified in the input file. All parameters are only limited by the amount of memory available. Selection, migration, and mutation are not included in the simulation model.

Each iteration starts by generating a founder population with the population size, observed number of alleles and allele frequencies specified by users. An age value is assigned to each individual; the degree of generation overlap specifies the percentage of individuals assigned a random age value within the limit of expected longevity. The age of individuals that are not assigned randomly is set to zero. When the degree of overlap is set to zero, all individuals start at age zero and reach the longevity limit in the same year, resulting in a complete population turnover equivalent to a discrete-generation model. When the degree of overlap is set to 100, all individuals in the population start with a random age value. The expected number of individuals in each age group is (N/L), where N is the founder population size and L is the length of expected longevity. Each simulation year includes two major temporal steps: (1) from previous year's end to current year's beginning, (2) from current year's beginning to current year's end. In the first major step, the program checks if population decline occurs and generates a list of surviving individuals. Age of all surviving individuals is increased by one. The second major step includes three sub-steps: (1) identify the individuals that reach reproductive maturity and generate a list of possible reproducing individuals, (2) identify the individuals that reach the longevity limit, replace them with new genotypes according to the reproductive system setting, and reset age to zero, and (3) check if population growth occurs and generate new individuals accordingly. All genetic diversity measurements are calculated at the end of each simulation year.

The output files generated by BottleSim are in ASCII text format with space delimiters, and can be viewed directly by word-processing software or imported into spreadsheet software for any further analysis. The summary output file contains all simulation settings, fixation probability, observed number of alleles (OA), effective number

of alleles (EA), observed heterozygosity (H_o), expected heterozygosity (H_e), and the fixation index ($F = (H_e-H_o)/H_e$). The optional genotypic data output file contains the raw genotypic data from the last year of each iteration. The genotypic data output is in GENEPOP (Raymond & Rousset 1995) format. Availability of raw genotypic data allows users to perform power analysis of statistical genetic tests.

To validate the simulation algorithm, we compared the simulation output to analytical expectation for the loss of heterozygosity (Fig. 1) and to another simulation program (England & Osler 2001) for the loss of alleles (Fig. 2) under the discrete-generation model. Excellent agreement was found under all simulation settings tested. All validating simulations were performed on an iMac with an 800MHz PowerPC G4 processor and 512MB of memory. Each of the validating simulations was completed within one minute. Fig. 3 demonstrates the flexibility of the program, including various settings of the degree of generation overlap (Fig. 3a), representative reproductive systems (Fig. 3b), and fluctuating population sizes (Fig. 3c). One example (Fig. 3c, ▲) also shows using the harmonic mean as the effective population size can lead to significant deviation when a population experiences cyclical changes in size (Motro & Thomson 1982).

The source code of BottleSim is written in C++ programming language. All the pseudorandom numbers used in the simulation process were obtained by using the rand() function in the C++ mathematical library. The random number generator function is seeded with the starting time of each run to ensure the uniqueness of each simulation. The source code, sample input files, users' manual, and compiled executables for Mac OS 9, Mac OS X, and Microsoft Windows operating systems are available free of charge at http://www.public.iastate.edu/~fjanzen/.

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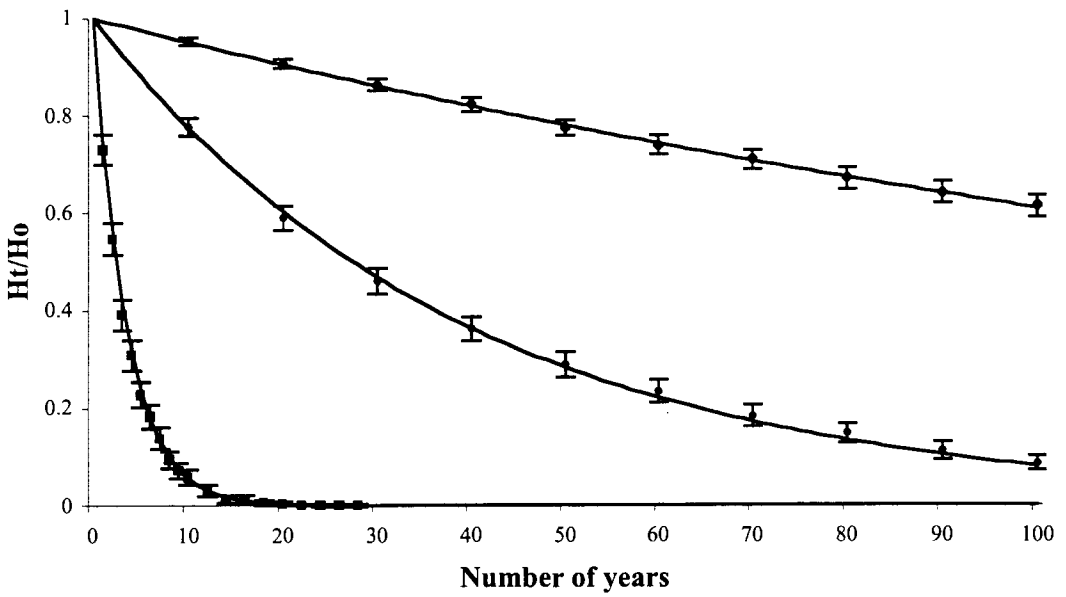
Fig. 1 Validating the loss of heterozygosity. a. Monoecy with random mating (with selfing). Solid lines show the analytical expectations of $H_t/H_o = (1-(1/2N_e))^t$, where H_t is the heterozygosity after t generations, H_o is the initial heterozygosity, and N_e is the effective population size. b. Monoecy with random mating (without selfing). Solid lines show the analytical expectations of $H_t/H_o = (1-(1/(2N_e+1)))^t$. In both a. and b., from top to bottom, curves show BottleSim projections from constant population size of 100 (◆), 20 (●), and 2 (■). Other simulation parameters were set as follows: degree of generation overlapping = 0, two alleles at equal frequencies, expected longevity = 1 year, age of reproductive maturation = 1, number of years simulated = 100, and number of iterations = 1000 (error bars show the 95% confidence intervals of iterations).

1a.


Ht/Ho
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
Number of years

1b.

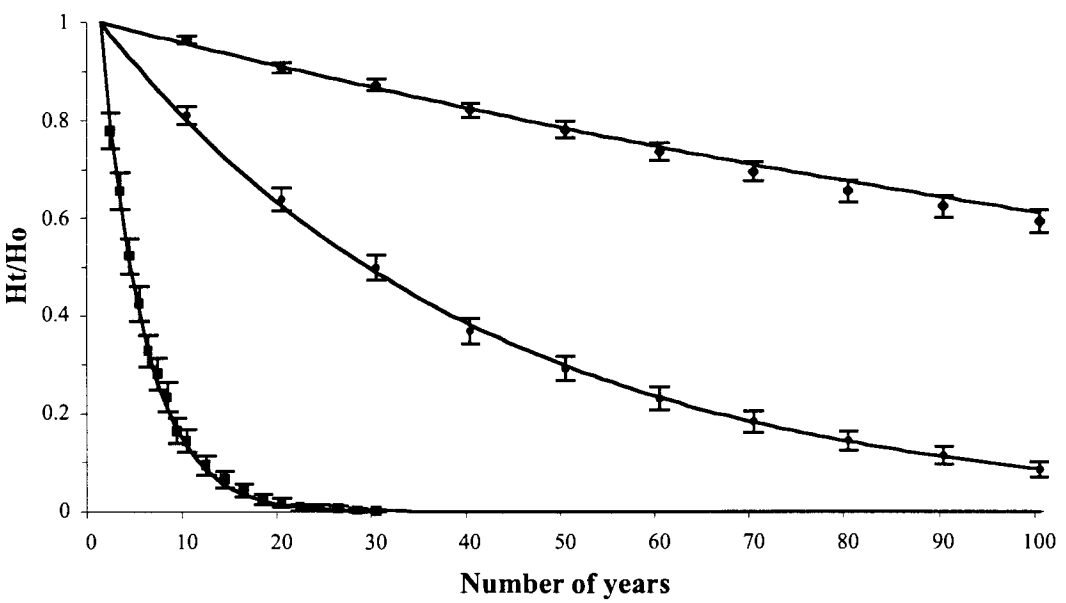
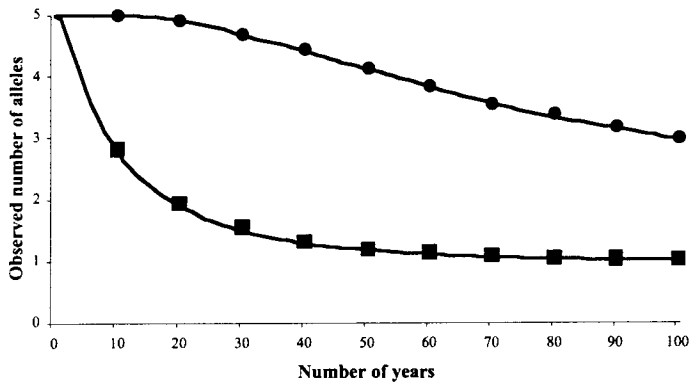
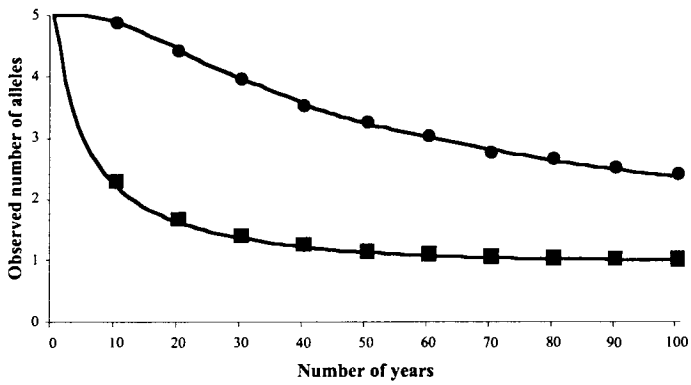

Ht/Ho
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
Number of years

Fig. 2 Validating the loss of alleles. a. Five alleles at equal frequencies. b. Five alleles at frequencies 0.6, 0.1, 0.1, 0.1, 0.1. c. 10 alleles at equal frequencies. Top curves show simulation result from a constant population size of 100 (BottleSim: solid lines, GENELOSS: ●). Bottom curves show simulation results from a constant population size of 10 (BottleSim: solid lines, GENELOSS: ■). Other simulation parameters were set as follows: degree of generation overlapping = 0, monoecy with random mating (with selfing), expected longevity = 1 year, age of reproductive maturation = 1, number of years simulated = 100, and number of iterations = 1000 (error bars indicating 95% confidence intervals are not shown because they are not discernible).

2a.


Observed number of alleles
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
90
100
Number of years

2b.


Observed number of alleles
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
90
100
Number of years

2c.

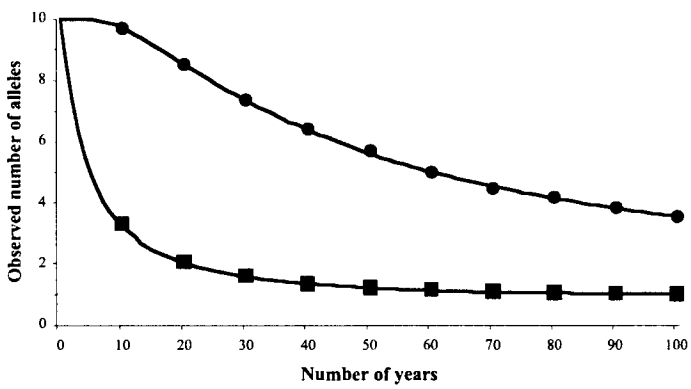
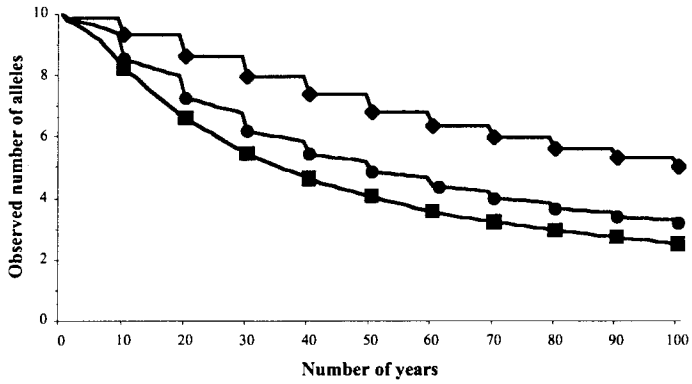
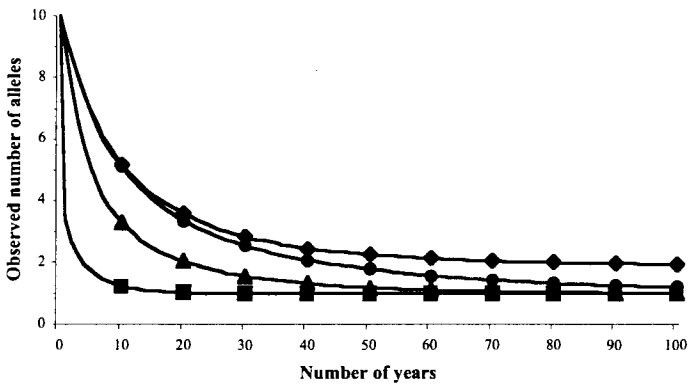

Observed number of alleles
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80
90
100
Number of years

Fig. 3 The loss of observed number of alleles under different simulation settings. a. Degree of generation overlap = 0 (◆), 50 (●), and 100 (■). Monoecy with random mating (with selfing), expected longevity = 10 year, age of reproductive maturation = 1, constant population size of 20. b. Representative reproductive systems: asexual reproduction (◆), monoecy with complete selfing (▲), dioecy with random mating (●), dioecy with single reproducing pair each year (■). Degree of generation overlap = 0, expected longevity = 1 year, age of reproductive maturation = 1, constant population size of 20. c. Fluctuating population sizes: 90 generations of population size =100 with 10 generations of bottleneck (population size = 10) in generation 1-10 (■), generation 46-55 (●), generation 91-100 (◆), and one bottleneck every 10 generations (▲). The smooth line shows a constant population size of 53 (approximation from harmonic mean). Degree of generation overlap = 0, monoecy with random mating (with selfing), expected longevity = 1 year, age of reproductive maturation = 1. Other simulation parameters were set as follows: 10 alleles at equal frequencies, number of years simulated = 100, and number of iterations = 1000 (error bars indicating 95% confidence intervals are not shown because they are not discernible).

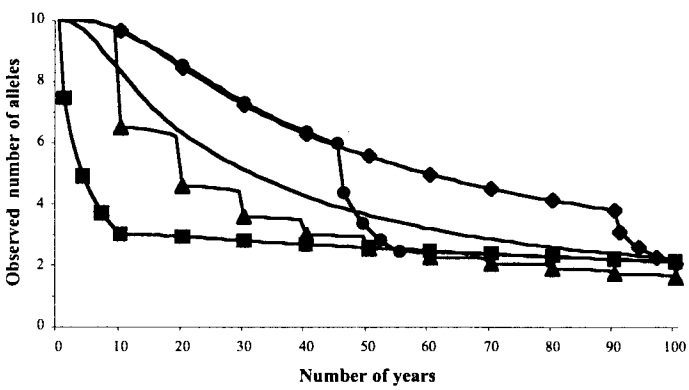
3a.


Observed number of alleles
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80
90
100
Number of years

3b.


Observed number of alleles
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80
90
100
Number of years

3c.


Observed number of alleles
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80
90
100
Number of years

APPENDIX II.

Microsatellite Loci List

Locus	Primer sequences (5'-3')
CmuA19	F: TAA GAG ACA GAT GCT CAG CAA G
	R: GTA CAT AAC ACG CAC CCA ATG
CmuB08	F: CTC TGA GAC CCT TAT TCA CGT C
	R: AGC CTTTGT CTG TAA GCT GTT C
CmuB12	F: TCA ATC TTC CAG CCT AAC TGT G
	R: AGG GAT GTG TTT TGC AAC TGG
CmuB21	F: CTA GTT CGA AAC AGG ACC GTT G
	R: CCA CAC GAC AGT TTG ATG TCA G
CmuB67	F: ACTCAA GCA CTG ACA CAC AAT C
	R: CCA GTA TTT GTG AGA ATT TCC TTC
CmuB91	F: TCA GGG AAG CAA TAG AAC ACT C
	R: TCT CAT CCC TAA GTA AAC CCA C
CmuD21	F: GCA GTT AGG CAT TAC TCA ACA TC
	R: AGG GTA TGA ATA CAG GGG TGT C
CmuD55	F: GTG ATA CTC TGC AAC CCA TCC
	R: TTG CAT TCA GAA TAT CCA TCAG
CmuD62	F: GGT GGT ATA GAA AAT CCT AAA ATG G
	R: GTG CAA ACT GTC TGG AAA TAG G
CmuD79	F: GCC CTG TTC CAT TCT TAT TCT G
	R: ATC CCC TTA GTC GTC TCT TTT C
CmuD87	F: AAA CCC TAA GAC ATC AGA CAG G
	R: CAA ATC CAG TAC CCA GAA AGT C
CmuD88	F: AAC AAT GCC TGA AAA TGC AC
	R: TAG GCT ACC TCT GAA AAT GCT G
CmuD90	F: ATA GCA GGA CAA TTA CCA CCA G
	R: CCT AGT TGC TGC TGA CTC CAC
CmuD93	F: AGA CTC TCT TGA CCA GAT TTT CTC
	R: TCT GCC TTC TAT CAC TCT CCT G
CmuD95	F: TAC GAG ACA GGA CAA AGT G
	R: TGA ATG CAG TGT AAC ATT TGA G
CmuD121	F: GGC AAA TAT CCA ATA GAA ATC C
	R: CAA CTT CCT CGT GGG TTC AG

Table 1. Primer sequences.

Locus	Repeat motif	Dye	Allele range (bp)
CmuA19	(CT)7(CA)14	TET	121-156
CmuB08	(CAT)10	TET	194-212
CmuB12	(CAT)7	TET	184-190
CmuB21	(CAT)10	TET	N/A
CmuB67	(TGA)13	TET	148
CmuB91	(ATG)6	TET	143
CmuD21	(TAGA)15	6-FAM	149-173
CmuD55	(GAGA)(CA)(GAGA)3	6-FAM	170-227
CmuD62	(GATA)11	TET	N/A
CmuD79	(GATA)10(AAT)2(CTGG)(AAT)(AT)5	TET	159-163
CmuD87	(GATA)3interrupt(GATA)22	6-FAM	192-250
CmuD88	(CTAT)18	6-FAM	102-163
CmuD90	(GATA)9	6-FAM	113-149
CmuD93	(CTAT)2interrupt(CTAT)18	6-FAM	347
CmuD95	(GATA)17	6-FAM	137-177
CmuD121	(GATA)8	6-FAM	129-166

Table 2. Repeat motif, fluorescent dye labeled, and allele range (bp).